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Comment

The Importance of Validating the Demethylating Effect of 5-aza-2′-deoxycytidine in Model Species

(A Comment on Cook et al., "DNA Methylation and Sex Allocation in the Parasitoid Wasp *Nasonia vitripennis*")

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abstract: The use of DNA demethylating agents has been popular in epigenetic studies. Recently, Cook and colleagues, in a 2015 *American Naturalist* article, claimed an effect of 5-aza-2′-deoxycytidine (5-aza-dC) on the sex ratio of a parasitoid wasp without verifying its effect on DNA methylation. We repeated the 5-aza-dC feeding treatment to test its effectiveness. We used bisulfite amplicon sequencing of 10 genes that either were heavily methylated, previously showed a response to 5-aza-dC, or were suggested to regulate fatty acid synthesis epigenetically, and we demonstrate that wasps fed 5-aza-dC did not show reduced DNA methylation at these loci. Therefore, the conclusion that demethylation shifts sex ratios upward needs reconsideration.

Keywords: bisulfite amplicon sequencing, DNA methylation, sex ratio, fatty acid synthesis, parasitoid, *Nasonia vitripennis*.

Introduction

Epigenetics is a rapidly expanding field of research. In an increasing number of developmental, reproductive, and behavioral processes, epigenetic variation has been shown to affect the phenotype and its plasticity (Bossdorf et al. 2010; Lyko et al. 2010; Duncan et al. 2014; Verhoeven et al. 2016). Epigenetic DNA modifications affect the accessibility of genomic regions for regulatory proteins or protein complexes through mechanisms such as altered DNA methylation patterns or altered histone modification. A large number of studies have reported variation between populations in the overall degree of DNA methylation patterns, which were linked to phenotypic differences (Hu and Barrett 2017). Also, the use of demethylating agents such as 5-aza-2′-deoxycytidine (5-aza-dC) has been popular in showing the impact of DNA methylation on the phenotype. The 5-aza-dC is incorporated into the DNA, where it inhibits DNA methylation (Momparler 1985), as has been shown in plants (Bossdorf et al. 2010; Vergeer et al. 2012), invertebrates (Vandegehuchte et al. 2010; Amarasinghe et al. 2014; Athanasio et al. 2018), and vertebrates (Christman 2002; Dasmahapatra et al. 2017).

Recently, Cook et al. (2015) applied 5-aza-dC to test for an effect of DNA demethylation on the sex ratio response of the parasitoid wasp *Nasonia vitripennis*, which is known to adjust the sex ratio of its clutch based on the number of founding females in a host brood (Hamilton 1967; Werren 1980). In this experiment, wasps that were fed 5-aza-dC in a 20% sucrose solution showed a slight but consistent shift in sex ratio in the direction predicted by theory. However, no validation of the actual demethylating effect of 5-aza-dC was carried out. In fact, demethylation efficacy should be verified for each organism and each concentration at which 5-aza-dC is applied because 5-aza-dC is known to have a wide range of antimetabolic activities that could confound its demethylating effect (Christman 2002). Therefore, the lack of such evidence in the study by Cook and colleagues precludes the observed change in sex ratio in *N. vitripennis* to be attributed to a decrease in DNA methylation.

Several other studies followed Cook et al. (2015) and administered 5-aza-dC in an identical manner to the same or other parasitoid species, with no (Sak 2017) or very limited (Pegoraro et al. 2016) quantification of its effect on DNA methylation. In the latter study, only four CpG sites were tested, each one in a different gene. Although subtle changes were seen in methylation status for three out of the four loci, loci were not tested separately for significant changes, and

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the number of CpG sites tested was too low to obtain a representative estimate of the effect of 5-aza-dC on the epigenome (Pegoraro et al. 2016).

Here we want to emphasize the importance of validating the demethylating effect of 5-aza-dC when testing hypotheses about the relationship between DNA methylation and phenotypic expression. In addition to sex allocation (Cook et al. 2015) and induction of diapause (Pegoraro et al. 2016), several other phenotypes have been implied in parasitoid wasps to be regulated epigenetically, including caste determination (Shaham et al. 2016) and fatty acid composition (Sak 2017). For a more reliable interpretation of all experiments with orally administered 5-aza-dC to parasitoids, it will be necessary to have a better idea of the extent and pervasiveness of demethylating effect of this method.

Therefore, we applied oral 5-aza-dC treatment on *N. vitripennis* in the same dose as previous studies (Cook et al. 2015; Pegoraro et al. 2016) and tested its effect on DNA methylation of 155 CpG sites distributed over 10 different genes. We included (i) genes that were heavily methylated based on several published genome methylation scans in this species (Wang et al. 2013; Beeler et al. 2014; Pegoraro et al. 2016), (ii) genes that were previously shown by quantitative polymerase chain reaction (PCR) to change their CpG methylation status in response to 5-aza-dC (Pegoraro et al. 2016), and (iii) genes that are implied to be regulated epigenetically based on the lack of sequence variation despite different expression levels and phenotypes (Visser et al. 2012; Buescher et al. 2013; Lammers et al. 2019). For the latter category, we chose genes that are involved in the fatty acid synthesis pathway, as *N. vitripennis* lacks the ability to convert dietary sugars into fatty acids (Rivero and Casas 1999; Visser et al. 2012), similar to most other parasitoid wasps (Ellers 1996; Visser and Ellers 2008; Visser et al. 2010). No apparent gene loss or pseudogenization has been observed in pathways relevant to lipogenesis (Werren et al. 2010), and DNA methylation has been suggested as a regulator of fatty acid synthesis in *Drosophila melanogaster* (Buescher et al. 2013). Therefore, we hypothesize that *N. vitripennis* may regain lipid production after demethylation.

Methylation status of the genes was assessed using high-throughput bisulfite amplicon sequencing (BSAS) on an Illumina platform (Masser et al. 2015). With this method it is possible to accurately estimate the percentage of methylated CpGs per site at very high coverage (on average >2,000 times per sample in our experiment). We tested the hypothesis that 5-aza-dC treatment following established methods in *N. vitripennis* (Cook et al. 2015) results in significant DNA demethylation.

In addition, we measured lipid reserves of wasps in control and 5-aza-dC treatments over a 9-day period. These phenotypic measurements served a twofold purpose: first, if 5-aza-dC treatments result in significant changes in DNA methylation, we can test whether there is a concomitant change in lipid levels. Second, if no effect of 5-aza-dC treatments on DNA methylation is found, the phenotypic measurements allow us to ascertain the general effectiveness of our 5-aza-dC treatment, as the antimetabolic activity of the demethylation agent is expected to cause a decrease in physical condition of the wasps, for which lipid reserves are a general and sensitive measure (Ellers 1996; Jervis et al. 2008).

Methods

Wasps, Feeding Treatments, and Lipid Measurements

The fully homozygous wild-type strain AsymCX of *Nasonia vitripennis* of which the genome sequence is available (Werren et al. 2010) was chosen for our experiment. These wasps were reared on *Calliphora vomitoria* pupae at 25°C, 16L:8D photoperiod, and 75% relative humidity. To test the effect of 5-aza-dC on *N. vitripennis*, newly emerged females (<4 h old) were randomly assigned to either of two treatments: 5-aza-dC and control. Each treatment consisted of four biological replicates, with each replicate consisting of 10 pooled females. Wasps were kept in a plastic tube (height × diameter = 75.0 mm × 23.5 mm) with a sponge stopper. Wasps in the control treatment were fed a 20% sucrose solution, while the wasps in the 5-aza-dC treatment were fed a 20% sucrose solution to which 10 μM of 5-aza-dC was added, following the methods applied by previous studies (Cook et al. 2015; Pegoraro et al. 2016). In both treatments, wasps were kept without food for several hours before giving them access to the sucrose solution to ensure a high motivation to feed. Wasps were observed to start feeding immediately when food was offered. This is important because 5-aza-dC has low solution stability and a half-life of about 25 min in human cytoplasma due to high activity of cytidine deaminase (Momparler 1985), an enzyme for which the gene is also present in *N. vitripennis* (LOC107980937). After 2 days, wasps were put in 70% ethanol and stored at −20°C for further analysis. No individuals were removed or excluded from the experiment because all survived our treatments.

As a positive control of 5-aza-dC effectiveness, another set of wasps was given the same feeding treatments as above in parallel. After feeding for 2 days on the same solutions as above, these wasps were given clean sucrose solution for another week to test for an extended effect of 5-aza-dC incorporation. Randomly chosen samples of wasps from both treatments were frozen on days 0 (emergence), 2, and 9. We measured their per capita lipid levels following standard methods (David et al. 1975; Ellers 1996; Visser et al. 2010). All data on lipid levels have been deposited

in the Dryad Digital Repository: https://dx.doi.org/10.5061/dryad.d305qm1 (Ellers et al. 2019).

DNA Isolation and Bisulfite Conversion

All four biological replicates (each consisting of 10 wasps) were washed with 70% ethanol, vacuum dried, and crushed in 100 μL of phosphate-buffered saline. For DNA isolation, we followed a modified protocol of Promega. In short, we added 100 μL of Nuclei Lysis Solution (Promega) and 2 μL of Proteinase K (20 mg/mL; Roche) and incubated the lysate for 60 min at 60°C. After incubation, we added 170 μL of Lysis Buffer (Promega), vortexed shortly, and centrifuged the mixture for 10 min at 14,000 rpm. We used spin columns and DNA wash buffer (Promega) to isolate the DNA from the supernatant. For each sample, an aliquot of DNA was converted with bisulfite following the protocol of the EZ DNA Methylation-Gold kit (Zymo).

PCR and Amplicon Cleanup

We selected 10 different gene fragments (table 1) for measuring the DNA methylation level. Genes *aa* and *rnapol* were chosen based on their consistently heavy methylation pattern in both Wang et al. (2013) and Beeler et al. (2014). Genes *perq* and *wdr36* contain highly methylated sites in the study by Pegoraro et al. (2016) and had reduced DNA methylation in their *dmnt3*-knockdown experiments. Genes *acc*, *fabd*, *fasn1*, *fasn2*, *fasn3*, and *mcd* are part of the fatty acid metabolic pathway, which has been suggested to be epigenetically regulated (Visser et al. 2012; Buescher et al. 2013).

For every fragment, we developed primers on the bisulfite-converted sequence. Furthermore, we added nonconverted PCR products of *aa* and *rnapol* to counterbalance the low CG content in the sequencing library due to bisulfite conversion of C → T. Primers and PCR conditions are listed in table 2. For PCR, we used the GoTaq Flexi DNA Polymerase protocol (Promega) according to the manufacturer's specifications. PCR products were loaded on a 1.5% agarose gel, and bands of the correct size were cut out. PCR fragments were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).

Amplicon Sequencing

DNA concentrations of all 96 (two treatments, 12 amplicons, four replicates per amplicon) purified PCR fragments were measured with Qubit DNA-HS technology (ThermoFisher Scientific). Amplicons were diluted to a concentration of 0.2 ng/μL. We prepared dual index libraries following the Nextera XT DNA Library prep kit and the Nextera Index kit (Illumina). Ninety out of 96 samples passed our quality check on an Agilent 2100 Bioanalyzer system, and these were normalized and pooled to a final pool of 3 nM. The pooled amplicon library was sequenced at Macrogen using Illumina MiSeq 150 bp paired-end sequencing technology. No reads mapping to *fasn2* were recovered, indicating that the library preparation failed for this amplicon. The raw short-read sequence data are available in the National Center for Biotechnology Information Short Read Archive under BioProject number PRJNA52613.

Data Analysis

Differences in lipid levels of wasps were tested using ANCOVA with feeding treatment and time as independent variables and fat-free dry mass as the covariate. ANCOVAs were used for every day separately as post hoc tests (with $\alpha = 0.05/3$) to verify on which day the lipid levels diverged between treatments. Raw sequence data were checked for quality with FastQC version 0.11.3 (Andrews 2010). Illumina primers were trimmed with Trimmomatic version 0.35 (Bolger et al. 2014) and demultiplexed with a Python script. Reads were mapped to target gene sequences using Bowtie 2 version 2.2.6 (Langmead and Salzberg 2012), and single-nucleotide polymorphisms (SNPs) were called using the mpileup function in SAMtools version 0.1.19 (Li et al. 2009) and summarized using BCFtools. Because we

Table 1: Overview of all 10 genes selected in our study

Gene	Locus identifier	Full name
aa	LOC100123627	Putative sodium-coupled neutral amino acid transporter
rnapol	LOC100123857	DNA-directed RNA polymerase III subunit RPC7-like
perq	LOC100117390	PERQ amino acid–rich with GYF domain-containing protein
wdr36	LOC100679400	WD repeat-containing protein 36
acc	LOC100123347	Acetyl-CoA carboxylase
fabd	LOC100680337	Probable malonyl-CoA-acyl carrier protein transacylase
fasn1	LOC100121447	Fatty acid synthase
fasn2	LOC100122099	Fatty acid synthase–like protein
fasn3	LOC100122083	Fatty acid synthase–like protein
mcd	LOC100120093	Malonyl-CoA decarboxylase

Table 2: Overview of all primers used in this study

Amplicon	Length (bp)	Annealing temperature (°C)	Cycles	Primer F (5′–3′)	Primer R (5′–3′)
AA-N	261	52	35	GGCAAATTGTGGCGATGTTA	CGACGCAGATTCCACAAAAA
AA	324	56	35	GGTAAATTGTGGCGATGTTATAAAT	GACGCAAATTCCACAAAAAAAT
RNApol-N	238	50	35	AGCTCGAAAAGAAGGAGACTACT-GC	TCCTCCACATCCTTGTCTTCAGA
RNApol	324	53	40	AAGGATGACGATGAGTTTGAAGA-TAA	CATAATCCGTACCCTCGTCCAT
PERQ	366	47	40	TTAGAAAGTGAAAGGTTTTAT	AACACCAATTAATTCTACTC
WDR36	400	55	40	YGGATTTTTTATTTTTATGTATTT-TTGAAAG	CRAAATAAACTAAAAACTTAACAAAATC
ACC	375	56	40	GAGAGAGTTTTTGGGGTGATA	AATTCTTTCAAAACAATAACAAAATTC
FabD	392	52	40	GGTTTAGTTGAATTGTAGTT	CATCACCTACTTTAATAACTC
FASN1	369	52	40	TGTGGGAGGTATTAGATAAATGG-AT	TAACCTACAATTTTTCACCAACCAA
FASN2	381	57	40	TTAAGTAGTTGGAAAAGGATTTGG	ACCTAAAATACACTCCTAAATTTACCTA
FASN3	376	51	40	TTTTTGGAATTTGGGGGAAG	AACAAAACCATAACCAACCTCTACATTT
MCD	399	49	40	AAAAGAGGTGATATATAATTTAA	CTTAATAATAAATCAACCATCC

used an entirely homozygous strain (AsymCX), SNP calling of reads against the reference genome gives only sites with varying methylation level plus a small number of sequencing errors. Efficiency of the bisulfite conversion was measured on the proportion of C → T SNPs in non-CpG sites. SNPs in non-CpG sites were removed from this data set. Only CpG sites with >100 times the coverage were retained. We calculated the proportion of methylated cytosines per site and plotted the results in R version 3.2.5 (R Development Core Team 2015). Using this pipeline, we obtained a data set of per-site methylation proportions for all amplicons and treatments. Differences in methylation level per site were tested separately for each amplicon using a two-way ANOVA with treatment and site as factors, including interactions. It is important to emphasize that site was included as a fixed effect in the model with each site assigned a unique code, so we did not test for a nonzero slope.

Results

The lipid content of wasps is plotted in figure 1. Despite feeding on sucrose, lipid levels decreased continuously over time in both treatments (ANCOVA, $F_{1,219} = 102.3$, $P < .001$), which conforms to expectations for fatty acid auxotrophs. Feeding treatment had a significant effect on lipid levels (ANCOVA, $F_{1,219} = 6.657$, $P = .011$), with a larger decrease in lipid content in the 5-aza-dC feeding treatment. On day 0, there was no difference between lipid levels between treatments (ANCOVA, $F_{1,74} = 0.907$, $P = .344$), while on day 2 (ANCOVA, $F_{1,69} = 6.775$, $P = .011$) and day 9 (ANCOVA, $F_{1,72} = 14.91$, $P < .001$) lipid levels were significantly lower in the 5-aza-dC feeding treatment than in the control treatment.

After quality filtering and trimming, the DNA sequence data set consisted of 1,513,412 paired-end reads in total. Of these, 90.8% mapped to the reference gene sequences. We obtained 2,101 SNPs in total, of which 1,357 had a coverage of >100 times per sample. Bisulfite conversion was highly effective at a rate of 98.86% ± 0.39% (mean ± SE). No C → T SNPs were observed in the non-bisulfite-converted amplicons. Figure 2 shows a histogram of the coverage per site for all amplicons combined. Of the 155 CpG sites of our target amplicons, 148 were SNPs in our data set. Some CpG sites had a lower proportion of cytosine than predicted by the bisulfite conversion efficiency (i.e., these sites are probably never methylated in situ).

The proportion of methylated cytosines is plotted per CpG site per amplicon in figure 3. For a substantial proportion of the 155 individual CpG sites, methylation level increased or decreased in the 5-aza-dC relative to the control, but we tested changes in methylation status at the whole amplicon level (table 3). In all amplicons we found significant differences in methylation level per site over the length of the amplicon (all $P < .05$). There were no significant differences in methylation levels between 5-aza-dC and control treatments (all $P > .1$) and no significant interactions between treatment and site on the amplicon (all $P > .05$).

Discussion

We expected 5-aza-dC-fed wasps to have a lower proportion of methylated CpG sites compared to the controls.

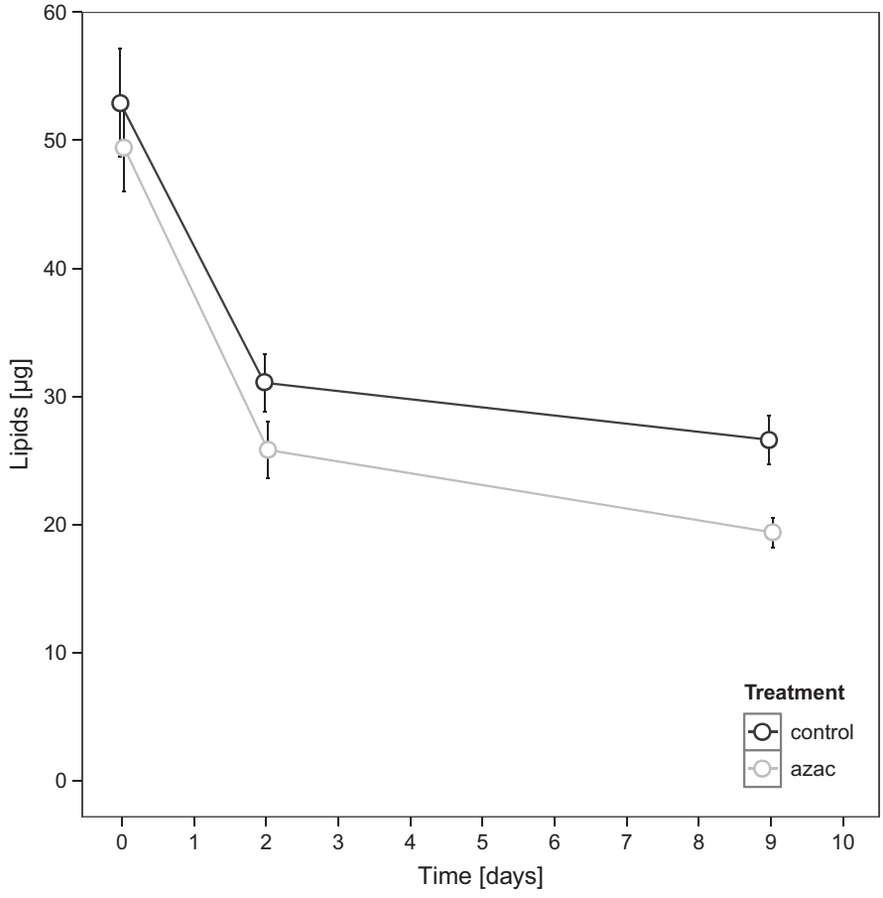

Figure 1: Lipid levels of wasps fed a 20% sucrose solution with (light gray line) or without (dark gray line) 10 μM of 5-aza-2′-deoxycytidine (azac) added.

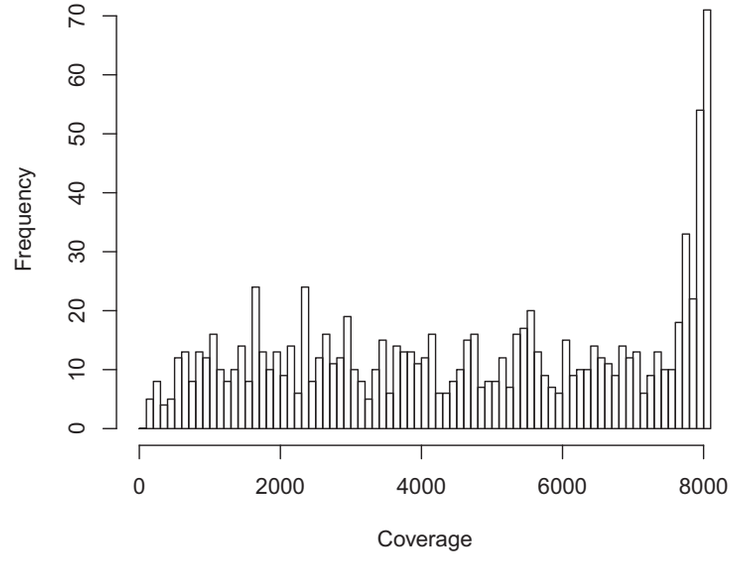

Figure 2: Histogram of read depth of each CpG site of the nine sequenced bisulfite-converted amplicons combined.

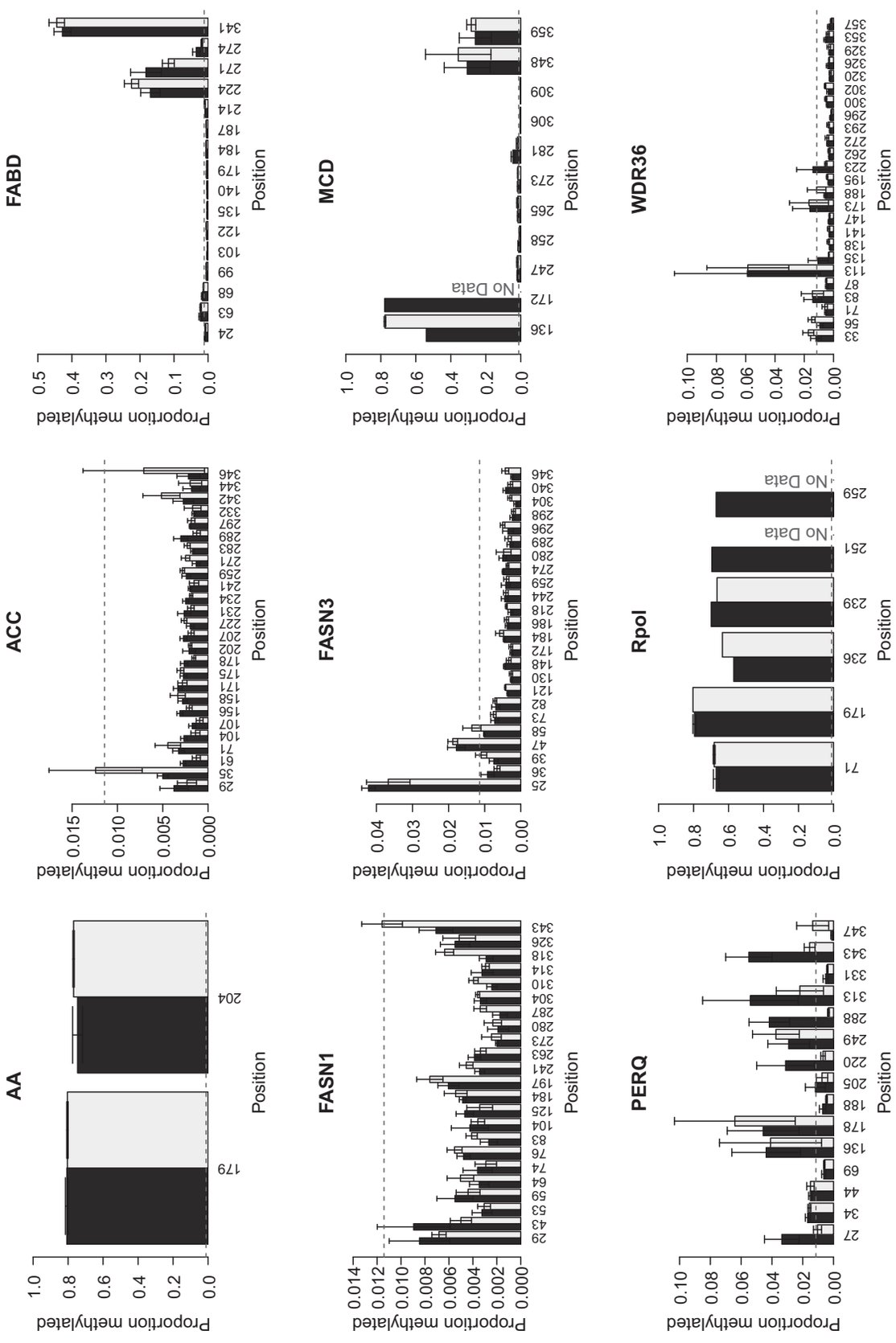

Figure 3: Proportion of CpG methylation (mean ± SE) per CpG site per sequenced amplicon. Dark bars represent 5-aza-2′-deoxycytidine-treated wasps; light bars represent controls. The *Y*-axes vary in range. Dashed horizontal lines at $Y = 0.0114$ represent the observed mean bisulfite conversion efficiency threshold.

Table 3: Effect of 5-aza-2′-deoxycytidine on the methylation level of nine amplicons

Amplicon and term	df	Sum of squares	Mean square	*F*	*P* (>*F*)
AA:					
Treatment	1	.00007	.00007	.086	.7779
Site	1	.007309	.007309	8.92	**.0203**
Interaction	1	.000442	.000442	.539	.4868
Residuals	7	.005736	.000819		
ACC:					
Treatment	1	4.6E-06	4.55E-06	.622	.43144
Site	25	.000416	1.67E-05	2.275	**.00123**
Interaction	25	.000202	8.07E-06	1.103	.34563
Residuals	156	.001142	7.32E-06		
FabD:					
Treatment	1	0	.00001	.024	.8783
Site	15	1.6226	.10817	177.234	**<2E-16**
Interaction	15	.0159	.00106	1.742	.0552
Residuals	96	.0586	.00061		
FASN1:					
Treatment	1	5.7E-06	5.68E-06	1.687	.1966
Site	22	.00051	2.32E-05	6.892	**1.09E-12**
Interaction	22	.000113	5.14E-06	1.526	.0787
Residuals	115	.000387	3.37E-06		
FASN3:					
Treatment	1	0	5E-07	.09	.765
Site	23	.008841	.000384	70.46	**<2E-16**
Interaction	23	.000128	5.6E-06	1.02	.446
Residuals	119	.000649	5.5E-06		
MCD:					
Treatment	1	.0073	.00732	.683	.413
Site	10	2.3903	.23903	22.308	**4.44E-14**
Interaction	9	.0403	.00448	.418	.919
Residuals	44	.4715	.01072		
PERQ:					
Treatment	1	.00195	.00195	2.567	.1133
Site	14	.02384	.001703	2.242	**.0133**
Interaction	14	.00798	.00057	.751	.7172
Residuals	75	.05696	.00076		
Rpol:					
Treatment	1	.00002	.000024	.067	.803769
Site	5	.05195	.010391	29.379	**.000379**
Interaction	3	.00242	.000808	2.285	.178931
Residuals	6	.00212	.000354		
WDR36:					
Treatment	1	0	2.7E-06	.008	.927851
Site	24	.0217	.000904	2.769	**.000132**
Interaction	24	.00042	1.75E-05	.054	1
Residuals	125	.04082	.000327		

Note: The two treatments tested were 5-aza-2′-deoxycytidine and control; site refers to the location of the methylation site in the amplicon. Site was significant for all amplicons, whereas treatment and interaction with site were not significant for any of the genes tested. Boldfacing indicates statistical significance.

However, no significant differences between 5-aza-dC-fed and control treatments were found in any of the genes we examined at the 10-μM doses provided, the dose commonly used in other studies in this model system (Cook et al. 2015; Pegoraro et al. 2016). Although for a substantial proportion of the 155 individual CpG sites we noted a decrease or increase in methylation status, our results demonstrate that 5-aza-dC does not consistently reduce DNA methylation in the nine amplicons that we tested. This result is corroborated by a recent genome-wide methylation

study in *Nasonia vitripennis*, also showing a lack of uniform demethylating effect of 5-aza-dC treatment across sites (Cook et al. 2019). We observed the same result for each of the three categories of genes we tested: there was no reduction in DNA methylation in highly methylated genes, nor in genes previously shown to have varying methylation levels, nor in genes involved in lipogenesis, a metabolic process that has been suggested to be epigenetically regulated. We found a negative effect of 5-aza-dC treatment on lipid levels of *N. vitripennis*, most likely due to the detrimental effects of antimetabolic activity of 5-aza-dC (Christman 2002). Therefore, previous studies that attributed phenotypic changes in 5-aza-dC-fed wasps to a decrease in DNA methylation should be reconsidered in the light of the current results.

The high proportion (98.86%) of C → T SNPs in non-CpG sites proved that the bisulfite conversion efficiency in our study was high, also compared to other methods (Olova et al. 2018). In CpG sites, the average methylation level was 0.09%–80.73% per site. These two observations imply that the lack of a demethylating effect of 5-aza-dC was not due to inefficient bisulfite conversion or improper detection of DNA methylation. Two CpG sites were tested in both our BSAS study and the MethylQuant-based study by Pegoraro et al. (2016). The MethylQuant-tested sites correspond in our study to sites 113 and 220 for *perq* and *wdr36*, respectively. Site 113 for *wdr36* is the only site in this amplicon with a detectable methylation level, with considerable variation between samples. Site 220 for *perq* seems to have a higher methylation level in 5-aza-dC-treated wasps. However, most other sites in this amplicon show no difference, similar to the rest of the data set. These differences could be life stage specific, a general pattern also found by Wang et al. (2013).

In plants and fungi, genome-wide DNA methylation levels have repeatedly been shown to decrease after 5-aza-dC treatment, often with associated changes in phenotypes such as growth, flowering phenology, or secondary metabolite production (Fieldes et al. 2005; Bossdorf et al. 2010; Vergeer et al. 2012; Wang et al. 2013). The evidence for a demethylating effect of 5-aza-dC in arthropods is much less strong. This is partly because the major insect genetic model *Drosophila* lacks a DNA methylation system (Bewick et al. 2017). In other arthropod model systems, DNA methylation is present. In *Daphnia*, for instance, 5-aza-dC had a clear demethylating effect on four out of the five tested genes, which differed among CpG sites (Athanasio et al. 2018). However, few insect studies have shown a consistent effect of 5-aza-dC. In bumble bees, 5-aza-dC has an effect on overall DNA methylation, but its effect is erratic, as hyper- and hypomethylation were both observed after 5-aza-dC treatment (Amarasinghe et al. 2014). A similar variable effect of 5-aza-dC treatment on methylation status of individual CpG sites was also found in a genome-wide study of methylation in *N. vitripennis* (Cook et al. 2019). Our study adds to this growing body of literature by showing a lack of consistent demethylating effect of 5-aza-dC in *N. vitripennis*.

There may be several causes for this lack of a demethylating effect of 5-aza-dC in *N. vitripennis* and in insects in general. The 5-aza-dC is usually administered via the food, and one explanation may be that the animals did not feed enough. In our study, however, we observed all wasps to feed within minutes after the food was offered, ensuring that 5-aza-dC was ingested by all wasps. Also, the phenotypic effect of 5-aza-dC treatment on lipid reserves indicates that the 5-aza-dC solution was effective. An alternative explanation for the lack of demethylating effect of 5-aza-dC could be that there is a paucity of cell division in adult insects (Lamb 1988). Incorporation of 5-aza-dC into the DNA happens only during cell division and is necessary to inactivate the DNA methyltransferase 1 enzyme (Christman 2002). In adult insects, only a few tissues contain regenerative cells, such as cells involved in neurogenesis in the brain of the house cricket *Acheta domesticus* (Cayre et al. 1994), intestinal stem cells in the midgut of *Drosophila melanogaster* (Jiang and Edgar 2011), and germ cells continuously dividing in ovaria of *N. vitripennis* (King and Richards 1969). Compared to organisms with continuous growth, the amount of cell division in adult insects is low, yet an effect of 5-aza-dC on DNA methylation would have been possible.

We showed a small but significant negative effect of 5-aza-dC on lipid levels in *N. vitripennis*. Lipid levels decreased over time in wasps in both treatments, but the rate of decrease was higher in 5-aza-dC-treated wasps. However, this is probably not biologically significant but due mainly to detrimental effects of 5-aza-dC on wasp health. The five genes involved in the fatty acid synthesis contain many CpG sites, but methylation levels varied substantially among genes. In FASN1, FASN3, and ACC, methylation levels at CpG sites were on average less than 1%, whereas MCD and FabD showed high methylation levels at CpG sites on various locations in the amplicon. However, 5-aza-dC did not influence the DNA methylation levels in any of these genes.

Our results suggest that phenotypic differences observed in adult wasps after administering 5-aza-dC arise through a mechanism independent of DNA methylation—for instance, an effect on other metabolic processes. Even though we did not quantify sex ratio in our study, our results warrant further investigation of the previously reported relationship between DNA methylation and sex allocation in *N. vitripennis* (Cook et al. 2015). Due to the inconsistent effect of 5-aza-dC for DNA demethylation, it would not be advisable for future studies on the link between CpG methylation and phenotypes in *N. vitripennis*. Pupal knockdown of DNA methyltransferases could be a good alternative, although this will also remove methylation of sites that could

be essential for successful metamorphosis and thus lead to premature mortality. A second alternative is the use of the nonspecific DMNT1 inhibitor RG108 (Biergans et al. 2017), but as far as we know this method remains untested in *N. vitripennis*. In all scenarios, the study should always include evidence showing consistent changes in DNA methylation.

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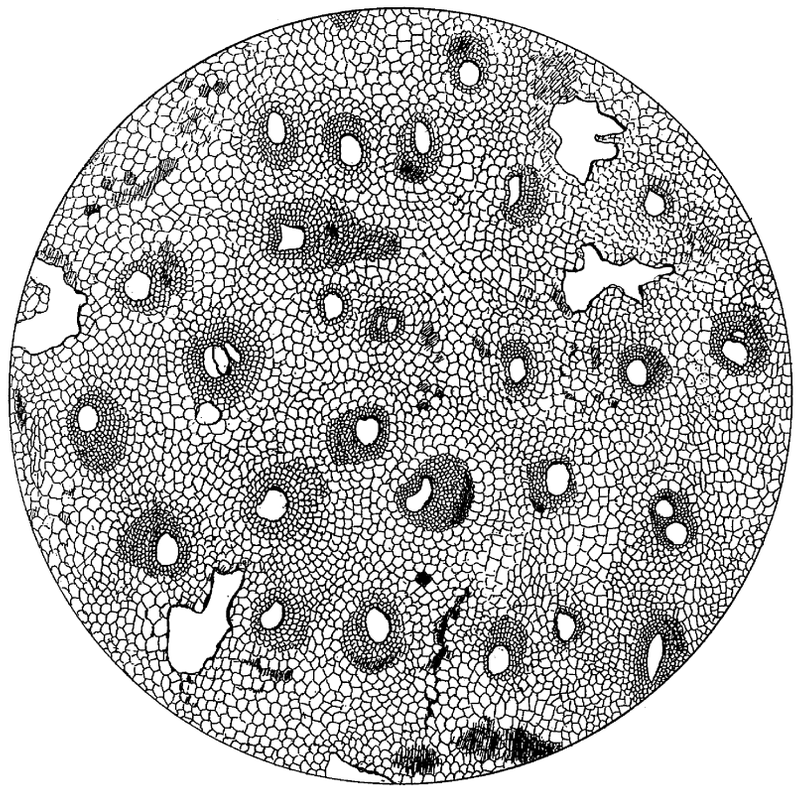

"All plants, it has been discovered, great and small, the monarch of the woodland and the violet of the plain; aye, all, with the exception, perhaps, of those doubtful little organisms that puzzle and delight the students of atomies, and which are grouped under the great collective head of *Protophyta*, are constructed after the same general plan, and consist of the same chemical substances, congregated together after similar types, varying only in degrees of complexity." From "Shavings Examined Microscopically" by A. M. Edwards (*The American Naturalist*, 1870, 3:561–568).